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Mutations in HIV-1 reverse transcriptase cause misfolding and miscleavage by the viral protease

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ABSTRACT

Previous work on mutations in the thumb of HIV-1 reverse transcriptase (RT) showed that the majority of the mutant RTs were degraded (by the viral protease) to various extents in virions. This degradation was, in most cases, temperature sensitive, and presumably was due to a partial unfolding of the protein at 37 °C.

We used recombinant proteins to investigate the effects of the mutations on the thermal stability and proteolytic degradation of RT.

Both subunits contribute to the stability of RT. In general, the differences in stability between the mutants and WT were greater if the mutation was in p51 rather than p66. Expressing the Pol polyprotein containing the RT mutants in *Escherichia coli* produced results similar to what was seen in virions; the mutant RTs were misfolded and/or degraded at 37 °C, but were better folded and processed at 30 °C.

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Introduction

The structural proteins found in mature HIV-1 virions are synthesized as parts of the Gag polyprotein; the viral enzymes are made as parts of the Gag–Pol polyprotein. Pol consists of protease (PR), reverse transcriptase (RT), and integrase (IN). Uncleaved Gag and Gag/Pol co-assemble with viral RNA and the envelope glycoprotein in producer cells to form the immature virion. Maturation, which is concomitant with virus budding, occurs when PR cleaves the Gag and Gag–Pol polyproteins into the structural components and enzymes found in the mature virion.

PR is a homodimer; dimerization is essential for PR activity. However, in an immature virion, PR is embedded within Gag–Pol. Although PR carries out the majority of the cleavages that are involved in maturation, the initial processing of Gag–Pol must either involve cleavages made by PR when it is still embedded in Gag–Pol, or be made by some unidentified host protease present in amounts too small to detect. PR is known to be one of the last proteins to be cleaved from Gag/Pol in an *in vitro* system (Pettit et al., 2005).

There are reports that either single (Huang et al., 2003; Takehisa et al., 2007; Wapling et al., 2005) or multiple point mutations (Huang et al., 1998) within RT destabilizes RT in virions, and mutations in RT could also have more global effects on the processing of Gag–Pol or Gag (Abram and Parniak, 2005; Huang et al., 2003; Olivares et al., 2007). We tested a number of mutations in RT, most of which are in the thumb subdomain, for their effects on the replication of a one-round vector. A large fraction (> 80%) of the mutations we tested affected the stability of RT in virions (Dunn et al., 2009). The mutant virions contained reduced amounts of intact p66 and p51, and for some of the RT mutants, the virions had no intact RT. The amounts of IN and PR, which are also derived from Gag–Pol in the virions, were normal, or nearly normal. We showed that the viral PR was responsible for the degradation of three of the RTs with mutations in the thumb; Wapling et al. (2005) showed that PR was responsible for the degradation of a connection subdomain mutant. Although, in our experiments, there was evidence that some of the mutations in RT caused a small portion of Gag–Pol to be misfolded and/or mis-processed, the fact that the mutant virions contained normal, or nearly normal, amounts of PR and IN suggested that the majority of the mutant Gag–Pol was appropriately processed and that RT was degraded by PR after it was properly cleaved from the polyprotein precursor. Several of the mutations conferred a temperature-sensitive phenotype on both viral replication and RT degradation; the triple-mutant described by Huang et al. (1998) was temperature sensitive.

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Because the fraction of the RT mutations that we tested that led to PR susceptibility was large, we also proposed that the susceptibility of RT to PR cleavage could be an important consideration for determining which RT mutations are and are not acceptable to the virus, an idea that has been supported by work from the Dykes laboratory (Wang et al., 2010).

We have developed *Escherichia coli* expression systems in which various forms of HIV-1 RT can be expressed and purified. These constructs can be used to express either the p66 subunit or p51 subunit by themselves, or a p66/p51 heterodimer in which only one of the two subunits is mutated (subunit selective expression). Another expression vector that contains both the p66 subunit coding region and a separately expressed protease coding region can be used to express and isolate p66/p51 heterodimers with the mutations in both subunits. The most recent construct, described here, expresses the entire Pol coding region in *E. coli* (see the "Material and methods" section), and is similar to the construct used by Wrobel et al. (1998). We show that the purified mature (p66/p51) form of each of the four mutant RTs, with mutations in both subunits, is temperature sensitive. Although the *E. coli* expression systems do not match the conditions either in an infected eukaryotic cell or in an assembled virion, the *E. coli* expression systems make it possible to dissect the effects of the mutations on RT stability in ways that cannot be easily accomplished using a eukaryotic viral expression system. In general, the mutant proteins behaved similarly in virions and in the *E. coli* expression systems: When the mutant RTs were expressed in *E. coli* as a component of Pol, their behavior was similar to what had been seen when the same mutant RTs were expressed as part of Gag–Pol and incorporated into virions, suggesting that the behavior of the mutant RTs in virions is due to the thermal stability of the mature mutant RTs, and to their interactions with PR.

Results

Because the degradation of RT was temperature sensitive in virions, we proposed that the mutations led to a partial unfolding of the protein, making the mutant RTs more susceptible to degradation by PR, and that the degree of unfolding was reduced at the lower temperature. This conjecture led us to investigate the behavior of recombinant versions of RT and Pol that carry the mutations that affect RT stability in virions and make viral replication temperature sensitive. We chose four mutant RTs to analyze (L264S, I274T, L279S, and L310S). All of these amino acids are located in the hydrophobic core of the thumb subdomain, and each of the four is in a position where it could contribute to the stability of the thumb (Dunn et al., 2009). As we previously proposed, replacing the hydrophobic side chains (I or L) that are normally present in the thumb with hydrophilic side chains (S or T) is likely to reduce the stability of the thumb. All four of the mutant RTs were extensively degraded in virions at 37 °C, and each of the mutants showed a considerable increase in amount of intact RT at 32 °C. As has already been mentioned, all of the mutant virions contained normal, or nearly normal, amounts of PR and IN; however, there were indications, in Western blots performed with anti-RT antibodies on the proteins present in virions, that the L279S mutation might have caused some misfolding and misprocessing of the Gag–Pol polyprotein (Dunn et al., 2009).

Thermal stability of the RT mutants

We purified recombinant heterodimeric (p66/p51) versions of wild-type (WT) HIV-1 RT and four of the temperature sensitive RT mutants (Boyer et al., 2001). We measured the thermal stability of these RTs using differential scanning fluorometry (DSF) in the presence of SYPRO Orange. Basically, the fluorescence of SYPRO Orange increases when it binds to hydrophobic groups. As the protein begins to melt, the hydrophobic groups normally found in the interior of the protein are exposed and are able to bind SYPRO Orange. Thus, measuring the level of SYPRO Orange fluorescence can be used to monitor the unfolding of a protein. In the presence of SYPRO Orange, the fluorescence of both WT and the mutant RTs increased as the temperature increased. Fig. 1A and Supplemental Fig. 1A show that the T_m for the RT mutants is about 4–5 degrees lower than WT. It is likely that the unfolding of the protein involves both the melting of the individual subunits, and the disassociation of the heterodimer. Because it is likely that it is the initial unfolding of the mutant RTs that makes them susceptible to degradation by PR, we also determined the temperature at which SYPRO Orange binding was first detectable (Fig. 1B and Supplementary Fig. 1B). Based on the results of the SYPRO Orange experiments, WT RT did not begin to unfold until the temperature reached 40–41 °C; however, all of the mutant RTs began to unfold below 37 °C. This nicely accounts for the fact that WT RT is stable in virions at the temperature at which the virus normally grows (37 °C), while the mutant RTs are susceptible to degradation under these conditions. Three of the mutants (L264S, L279S and L310S) showed what appeared to be two-stage melting (Supplementary Fig. 1A). In HIV-1 RT, a mutation in the polymerase domain leads to an amino acid change in both the p66 and the p51 subunits.

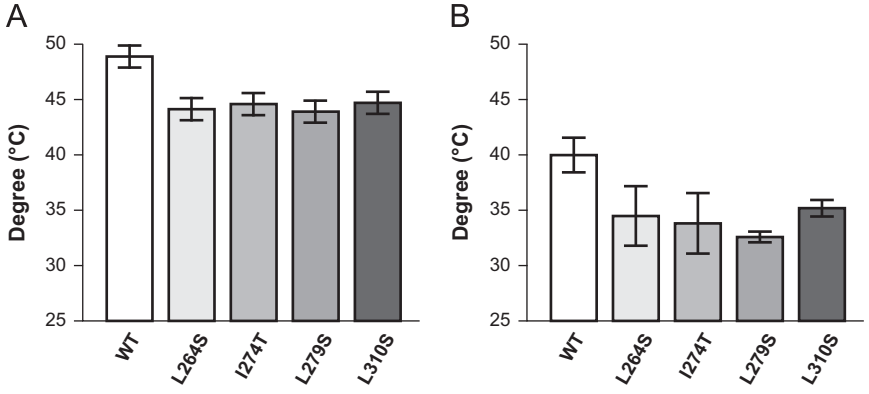

Fig. 1. The mutant RTs melt at a lower temperature than WT RT. Purified WT and mutant RTs were heated (1°/min) in the presence of Sypro Orange. The change in fluoresence that occured as the temperature was increased was monitored using a PCR machine (see the "Material and methods" section). Panel A shows the T_m, defined as the temperature at which the rate of change in flouresence is maximal (see Supplemental Fig. 1). The data for L264S are the average of of two experiments; the data for I274T, L279S and L310S are the average of three experiments and WT are the average of four experiments; the bars show the standard deviation. Panel B shows the temperature at which an increase in fluoresence could first be detected. The graph shows the average; the bars show the standard deviation (see also Supplemental Fig. 1).

Because the organization of the subdomains differs in the two subunits, it is possible that the two-stage melting of some of the mutants is the result of a differential effect of the mutation in the two subunits.

For two of the mutants, L264S and L279S, we investigated the contribution of the mutations in each of the two subunits of RT, p66 and p51, to the reduced stability of the mutant proteins. Versions of RT that had a mutation in only one of the two subunits were expressed in *E. coli* using a coexpression system based on a plasmid that separately encodes the two RT subunits. After expression, heterodimeric dimers were purified (Boyer et al., 1994). When the thermal stability of the RTs with a mutation in only one of the subunits was measured using SYPRO Orange DSF, we found that mutations in either subunit contributed to the instability of the dimer. Fig. 2A shows that a mutation in p66, in combination with WT p51, gave a 1.5–2 °C shift in the T_m, and Fig. 2B also shows that a WT p66 in combination with a mutation in p51 gave approximately a 2–2.5 °C shift in the Tm. The RTs that had a mutation in only one of the subunits showed less evidence of two-stage melting (Supplementary Fig. 2), although the mutant RT with the L279S mutation only in the p51 subunit showed some evidence of two-stage melting. The reduction in the two-stage melting might be the result of a differential impact of the mutation on the structure of the thumb subdomain, the interaction of the two subunits, and perhaps, the stability of the heterodimer (see Discussion).

Folding and processing of WT and mutant RTs in E. coli

Having demonstrated that the purified mutant RTs have a lower melting temperature than WT RT, we wanted to study the folding and processing of various forms of the mutant RTs in *E. coli*. One of the advantages of using *E. coli* to express recombinant RT proteins is that it is possible to express the viral RT/Pol proteins either in the presence or the absence of PR. Recombinant proteins produced in *E. coli* have several possible fates. The proteins can be synthesized, appropriately folded, and soluble in bacterial extracts (although, in some cases, even properly folded recombinant proteins can be cleaved by bacterial proteases). If a recombinant protein is improperly folded, it can be degraded by bacterial proteases and/or be directed into insoluble inclusion bodies. Several different forms of the RT proteins were expressed in *E. coli* grown either at 30 °C or at 37 °C. When the mutant RT proteins were expressed in *E. coli*, the mutations affected both stability and solubility of RT; the proteins were more stable and more soluble at 30 °C than at 37 °C. Mutations in either p66 or p51 contributed to a reduced melting temperature of the heterodimeric form of RT, and to a decrease in its solubility and/or stability in *E. coli*. When the mutant RTs were expressed as either p51 alone or p66 alone, which are either almost entirely monomeric (p51) or partially monomeric (p66) (Restle et al., 1990), both p51 and p66 were less soluble and more easily proteolysed than the corresponding p66/p51 heterodimers. Introducing the RT mutations into a Pol polyprotein expressed in *E. coli* mimicked the effect of these mutations in Gag–Pol expressed in eukaryotic cells; the mutant RTs derived from these Pol expression systems were more susceptible to degradation at 37 °C than at 30 °C.

We began with an expression plasmid that leads to the expression of the p66 subunit (Hizi et al., 1988). The *E. coli* were allowed to express either WT or the mutant RTs for 2 h. The bacteria were harvested, lysed, and the soluble and insoluble protein fractions were separated by centrifugation. When the bacteria were grown at 37 °C, most of the WT and mutant RTs were found in soluble fraction; however, particularly for L279S and L310S, a significant portion of the RT was found in the insoluble fraction, although there was relatively little evidence of degradation of the p66 form of the mutant RTs by bacterial proteases (Fig. 3, panel A). Growing the bacteria that expressed the p66 form of RT at 30 °C had relatively little effect on the proportion of the RTs in the soluble fraction. Fig. 3 also shows the results obtained when the p51 subunit was expressed by itself in *E. coli* (Panel B). Several of the mutant p51 subunits (in particular I274T) were more susceptible than WT to folding problems/degradation at 37 °C; however, reducing the temperature to 30 °C greatly increased the proportion of the mutant RTs that was soluble and undegraded.

We also co-expressed p66 and p51, using a subunit selective RT expression vector, to ask what effect having the WT form of the complementary subunit would have on the stability of a mutant subunit in *E. coli*. In contrast to the p51 subunit, which is essentially all monomeric, or the p66 subunit, which is a mixture of monomers and dimers, the p66/p51 heterodimer is quite stable (Restle et al., 1990). If only the p66 subunit carried the mutations, and the p51 subunit was WT, then most of mutant RTs behaved in a fashion that was similar to an RT in which both subunits were WT at both 37 °C and at 30 °C. At 37 °C, the L279S

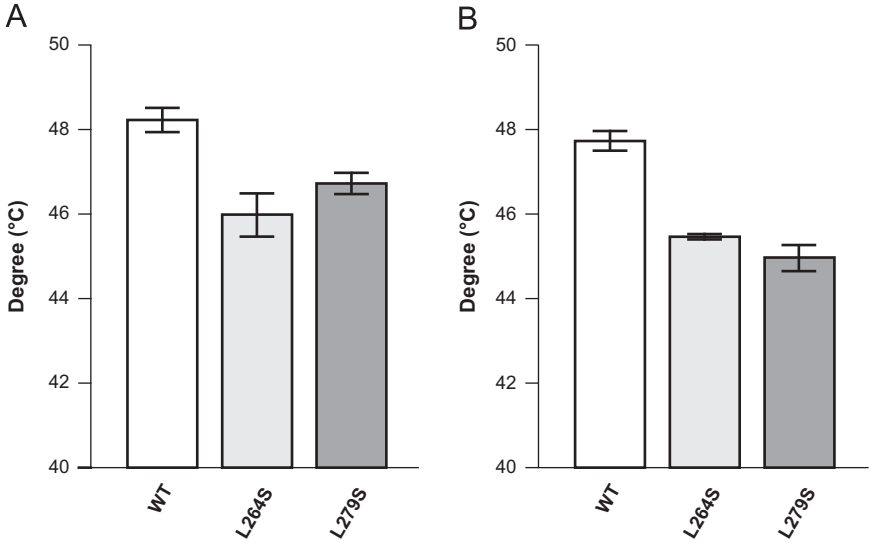

Fig. 2. Both subunits of RT contribute to the stability of the p66/p51 heterodimer. Purified RTs which have the specified mutation present in only one subunit were slowly heated in the presence of Sypro Orange. The analysis was done as described in Fig. 1. Panel A: RTs in which the p66 subunit was mutant and the p51 subunit was WT. The graphs represent the average of two experiments, the bars show the standard deviation. Panel B: p66 WT/p51 mutant (see also Supplemental Fig. 2).

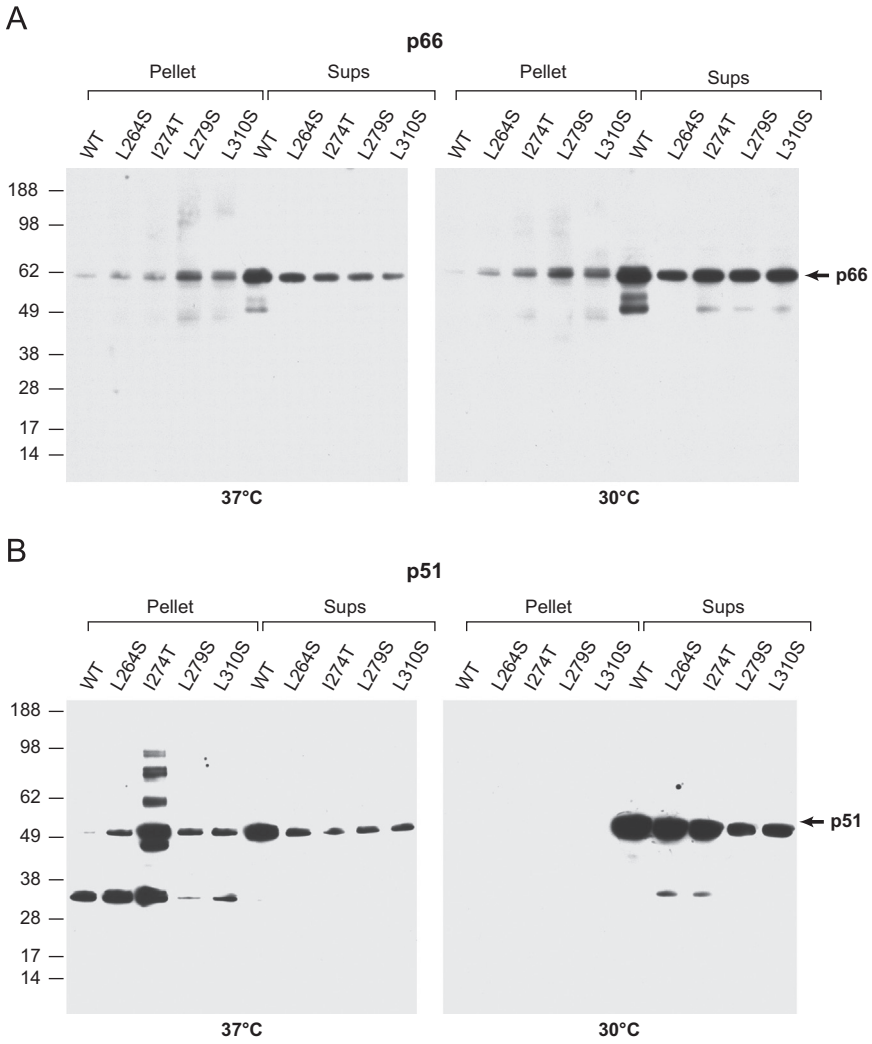

Fig. 3. The individual subunits of RT show some misfolding and degradation at 37 °C, but are more stable at 30 °C. RT subunits were expressed in *Escherichia coli* and the bacteria were harvested, lysed and the lysates centrifuged to produce insoluble and soluble fractions. The fractions were denatured in SDS and aliquots were fractionated by SDS-PAGE, transferred and Western blotted with an anti-RT mouse monoclonal antibody mix. Panel A: Solubility of the p66 subunits: Insoluble (pellets) and soluble (sups) fractions from bacteria grown at 37 °C and at 30 °C. Panel B: Solubility of p51 subunits: The data are similar to Panel A, except that the bacteria expressed p51.

and L310S mutants may have showed a modest increase in the amount RT in the pellet, and the L310S showed some evidence of degradation in the soluble fraction, but these effects were relatively minor. However, introducing the mutations into the p51 subunit had a much more dramatic effect, and this effect was particularly obvious at 37 °C. At 37 °C, all of the RTs with mutant p51 subunits are less soluble and more extensively degraded than WT. The heterodimeric proteins with mutations in p51 were much more soluble and less extensively degraded at 30 °C than at 37 °C, although there is some indication that the mutations in RT may have affected the amount of p51 in the pellets, even at the lower temperature. These data match the results we obtained when the two subunits were separately expressed: The mutant forms of the p66 subunit appear to be more stable and less susceptible to degradation at 37 °C than are the corresponding mutant p51 subunits. Because the subunit selective expression system should not produce any RT proteins larger than p66, the immunoreactive proteins that are larger than p66 seen in both the insoluble and soluble fractions in the bacteria grown at 37 °C are presumably the result of S–S crosslinking. The band seen in the soluble fraction is approximately the right size to be a crosslinked p66/p51 heterodimer; crosslinked S–S HIV-1 RT dimers are known to occur and usually involve crosslinks of C280 (Hizi et al., 1992) (see Fig. 4B).

Mutations in RT affect its susceptibility to PR in E. coli.

We expressed, in *E. coli*, a Pol polyprotein that contains WT RT and versions of Pol that contained three of the four temperature sensitive mutations in RT. We also expressed a version of Pol in which the active site of PR was mutated. The bacteria were grown at either 37 °C or 30 °C, harvested, lysed, and the lysates were separated into soluble and insoluble fractions. The proteins were solubilized in SDS, separated on SDS-PAGE and Western blots were prepared and probed with antibodies to RT, PR, and IN (Fig. 5). In the absence of PR activity, there was some processing of Pol, particularly at 37°, presumably by *E. coli* proteases; however, none of the processed products appeared to correspond to either of the normal subunits of RT, and none of these processed products were in the soluble fraction of the *E. coli* lysate (Fig. 5D). In the presence of PR activity the WT Pol polyprotein was processed at both 30° C and 37° C and both the p66 and p51 subunits were produced. Although essentially all of the WT Pol polyprotein was properly processed at 37 °C, the polyproteins that contained the mutations in RT were degraded and a significant portion of the degraded mutant RT proteins were insoluble (Fig. 5A). However, the mutant RTs we tested were properly folded and processed at 30 °C; at this temperature, essentially all of each of the mutant RTs were

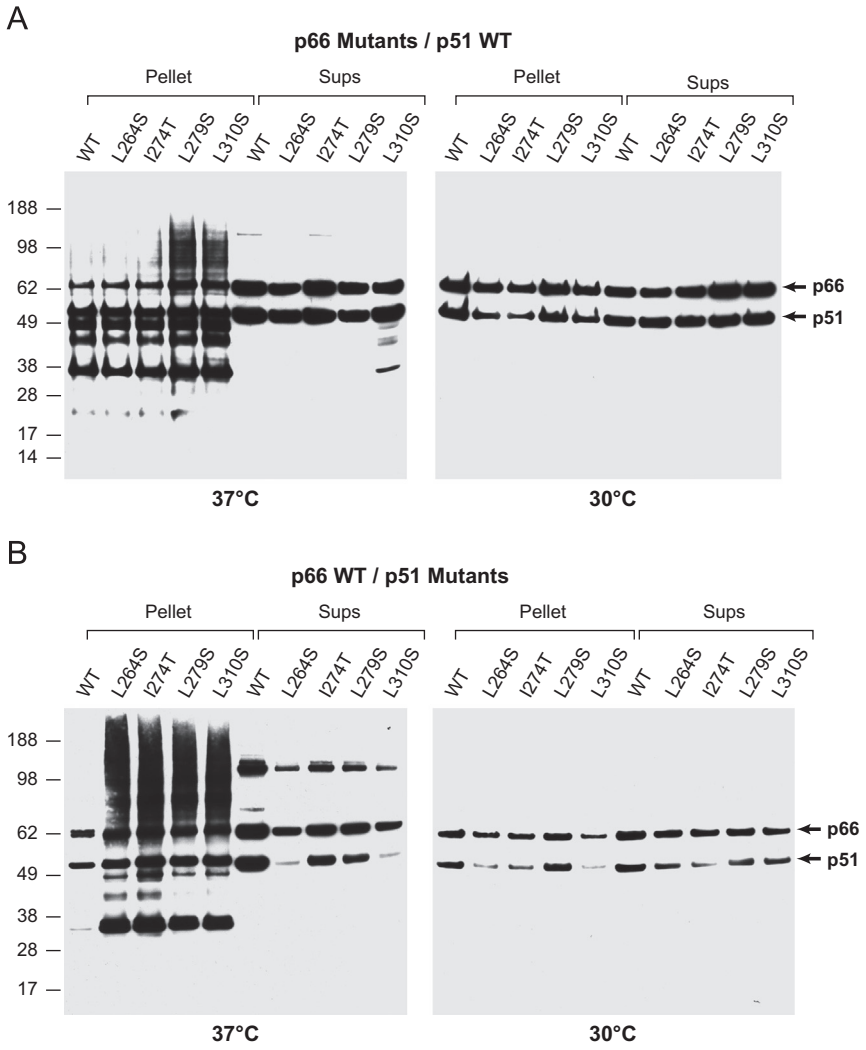

Fig. 4. Expression, in *E. coli*, of RTs that have a mutation only in the p66 subunit or only in the p51 subunit. A subunit selective expression system was used to express forms of RT with the mutations only in either the p66 or the p51 subunits. The bacteria were collected by centrifugation, lysed, and the lysates separated into insoluble and soluble fractions. The fractions were denatured in SDS, fractionated by SDS-PAGE, and the RT was detected by Western blot as described in Fig. 3. Aliquots were loaded on gels, transferred and Western blotted with an anti-RT mouse monoclonal antibody mix. Panel A p66 mutant/p51 WT. Panel B p66 WT/P51 mutant.

converted into soluble p66/p51 heterodimers. It would appear that, when Pol is processed by PR, that the p66 subunit of the L264S was more susceptible to degradation at 37 °C. This is in contrast to what was seen when the two subunits of RT were separately expressed in the absence of PR activity (Fig. 4). When the bacteria were grown at 37 °C, the amount of processed IN was similar for the WT Pol and for the mutant forms of Pol (Fig. 5C). There appeared to have been a modest increase in the fraction of IN that was soluble when the bacteria were grown at 30 °C compared to 37 °C. The IN antisera was polyclonal, and the antisera recognized *E. coli* proteins, one of which can be seen just below the IN band on the blot (Fig. 5C). The PR antiserum was a monoclonal and we were able get darker exposures (Fig. 5B).

When the mutant and WT bacterially expressed Pol proteins were analyzed for PR expression, solubility, and degradation, there was fully processed PR present in cells expressing both the WT and the mutants at both 37 °C and 30 °C. However, in contrast to what was seen with RT, the soluble and insoluble PR proteins looked quite similar at the two temperatures and the presence of the mutations in RT had only a relatively modest effect on the processing or solubility of PR. If anything, the bacteria expressing WT Pol seem to contain less soluble PR at both 30 °C and 37 °C. We have no simple explanation for this observation. In the soluble fraction, the presence of mutations in RT appeared to lead to the production of a modest amount of a protein that reacts with anti-PR antisera that was larger than mature PR. What was at first surprising, based on the data supplied by the manufacturer, that the PR monoclonal reacts only with mature PR, was the presence of bands significantly larger than mature PR in the insoluble fractions at both 37 °C and 30 °C. We considered the possibility that these were partially processed Pol proteins; however, the fact that the RT blot showed that there were no partially processed Pol proteins present that contain RT sequences at 30 °C showed that this was not the explanation [previous experiments with the RT antibody mixture showed that it can detect partially processed Gag–Pol proteins in Western blots (4)]. It appeared that the major PR related bands in the insoluble fractions are, in size, multiples of the PR monomer (dimer, trimer and tetramer); this is most clearly seen in the insoluble fraction for the WT sample at 37 °C (Fig. 5B). Mature PR contains two cysteines; we propose that, like RT, if PR is not properly folded that the cysteines it contains can lead to crosslinking (particularly in the insoluble fraction), which would explain the presence of bands larger than the PR monomer which are not recognized by RT antibodies. This explanation is supported by experiments in which strong reducing agents were added to the PR containing samples before the samples were fractionated on

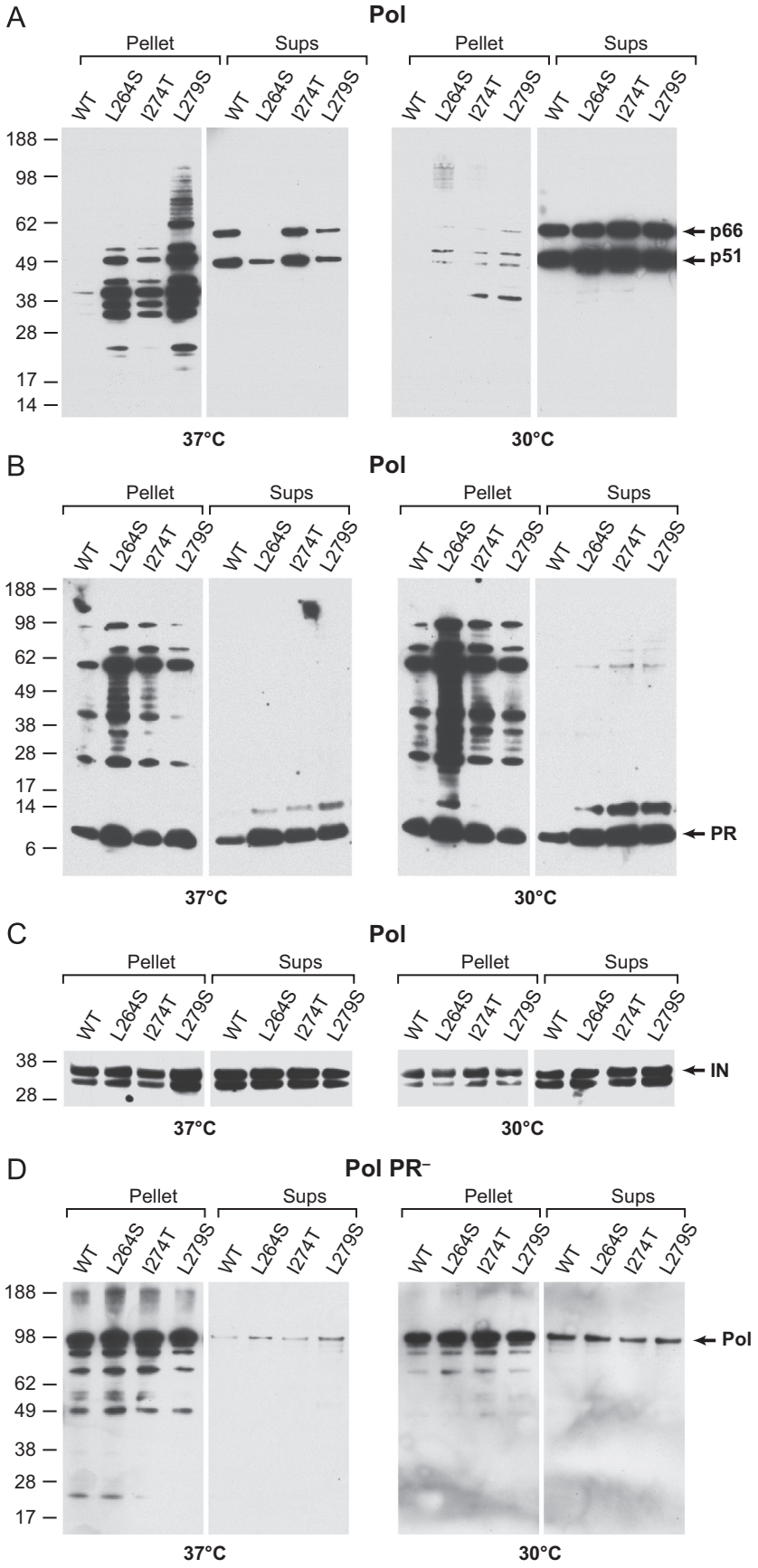

Fig. 5. IN and PR are appropriately cleaved from Pol in *E. coli*. Pol polyproteins that contained either WT or mutant RTs were expressed in *E. coli*. The bacteria were lysed and the lysates separated into insoluble and soluble fractions. Aliquots were denatured in SDS, fractionated on SDS-PAGE, transferred and Western blotted with antibodies that could detect RT (Panel A), PR (Panel B), and IN (Panel C). In the IN blot, the upper band corresponds to full length IN and the lower band corresponds to an *E. coli* protein detected by the rabbit polyclonal IN antiserum. The blots for RT (Panel A) gave a dramatically stronger signal for the soluble fraction at 30 °C (sups) so the exposure shown in the figure was made using two additional films to reduce the signal. The exposure was 3 s. The 37 °C pellet and soluble fraction blots and the 30 °C pellet blots were exposed for 5 s using one film to reduce the signal. Panel D shows a control experiment in which the Pol polyproteins containing either the WT or the mutant RTs and a mutation in the active site of PR were expressed at either 30 °C or 37 °C. The blot was incubated with anti-RT antibodies.

gels; this treatment reduced the amount of the larger immunoreaction PR bands, suggesting that they are the result of S–S crosslinking rather than incomplete digestion of Pol (data not shown). If this is the correct explanation, then the PR data supports the conclusion reached from the analysis of IN; in the presence of the mutations in RT, PR processing produces primarily mature IN and PR. This means that the *E. coli* expression system closely mimics what was seen in the virions where most of the degradation of RT by PR occurs after RT is cleaved from the precursor polyprotein.

Conclusion

Based on the increased susceptibility of the mutant RTs to degradation by PR in virions at 37 °C, and the fact that the mutant RTs were less susceptible to degradation at 30 °C, we predicted that the mutant RTs would unfold at a lower temperature than WT RT. Experiments with purified recombinant RT validated this conjecture, and experiments with RTs in which the temperature sensitive mutations were present in only one of the two subunits showed that having a mutation in either subunit destabilizes RT, although to a lesser extent than having a mutation in both subunits. Based both on the Sypro Orange melting experiments and the experiments in which the stability and solubility of the heterodimers was measured in *E. coli* at 37 °C, it would appear that having a mutation in p51 had a slightly greater impact than having the corresponding mutation in p66, at least for the mutants we tested. This is not simply a matter of these mutant p51s being unable to participate in the formation of a stable dimeric RT. When the *E. coli* host is grown at 30 °C, good heterodimers are formed, and good heterodimers can be formed at 30 °C even if both subunits contain these mutations. We suggest that having the mutations in the p51 subunit has a greater impact because the role of the thumb subdomain is different in the two subunits. The thumb of p66 helps position the nucleic acid substrate at both the polymerase and RNase H active sites, but the thumb of p66 plays no role in the interaction of the two subunits. In contrast, the thumb of p51 makes extensive contacts with the RNase H domain, and helps position RNase H so that it properly contacts and appropriately cleaves an RNA/DNA substrate (Sevilya et al., 2001, 2003). Although having one of the temperature sensitive mutations in p51 did not (by itself) interfere with the formation of a stable heterodimeric RT at 30 °C, if the temperature was raised to 37 °C, as the p51 thumb would begin to unfold; this would disrupt not only the structure of the mutant p51 thumb, it could also affect the structure of the p66 subunit and/or the association of the two subunits and the stability of the RT heterodimer. These different effects on the unfolding the p51 thumb could also help to account for the fact that there was evidence, at least for the L264S mutant, that having a change in only the p51 subunit of a heterodimeric RT could lead to a two-stage melting curve. In contrast, a partial unfolding of the p66 thumb could make the p66 subunit more susceptible to degradation; however, there is no reason to suspect that a partial unfolding of the p66 thumb would, by itself, affect the stability of the RT heterodimer.

We saw small amounts of misfolded or misprocessed Gag–Pol when virions in which the Pol gene carried the temperature sensitive RT mutations were grown at 37 °C (Dunn et al., 2009). Because the amounts of PR and IN were similar in these mutant virions to what is seen in virions that expressed WT RT, it would appear that a significant fraction of Gag–Pol was properly folded and incorporated into virions. However, it is possible that the presence of the mutation in RT led to the generation of some misfolded and or misprocessed Gag–Pol in producer cells, and that some, or all, of this misprocessed/misfolded Gag–Pol was not incorporated into viral particles. Only small amounts of Gag–Pol are synthesized in infected cells, and it is difficult to monitor the disposition and fate of Gag–Pol in infected cells. Wrobel et al. (1998) used a Pol expression system to study effects of mutation in the fingers and palm subdomains on the processing of Pol and the stability of RT at 37 °C. They identified several regions that appeared to be important for the stability of RT (124–133, 143–150, 167–169 and 187–191). We used a similar *E. coli* Pol polyprotein expression system to monitor the properties and fate of the Pol polyprotein. Knowing that the RTs we were studying were temperature sensitive, we studied their behavior at both 37 °C and at 30 °C. When the Pol polyprotein was expressed in *E. coli*, and the Western blots were analyzed with anti-RT antibodies, the presence of the temperature sensitive mutations in RT had a small, but measurable, effect on the processing of Pol at 37 °C, but not at 30 °C. Although a portion of the processed IN and PR was in the insoluble fraction at both temperatures, the fraction of the processed IN and PR proteins that was soluble did not appear to be greatly affected by the presence of the mutations in RT. The presence of the mutations in RT had a substantial effect on the solubility and degradation of RT at 37 °C. As described in the "Results" section, there were some differences in the degree to which the various mutations in RT affected the ability of RT to be expressed as a soluble heterodimer at 37 °C. Although we did not determine exact sites at which the mutant RTs were cleaved in virions, the pattern of RT fragments that were produced from Pol in *E. coli* at 37 °C appeared to be the same or very similar to the pattern that was seen when RT was degraded by PR in virions (Dunn et al., 2009): there was a degradation fragment slightly smaller than p51 and four smaller RT fragments ranging in size from about 30 kDa to about 42 kDa. This reinforces the idea that PR degrades the mutant RTs after they have been released from the precursors (Gag–Pol in virions and Pol in *E. coli*) and that the exact nature of the polyprotein (whether it does or does not contain Gag) is not critically important.

These results suggest that the behavior and processing of Gag–Pol carrying the RT mutants by PR in virions and Pol carrying the RT mutants by PR in *E. coli* are quite similar. This implies that, in both systems, the outcomes are due to the direct effects of the mutations in RT, and more particularly on the properties of mutant RTs after either Gag–Pol or Pol was processed by PR, and how RT interacts with PR. This conclusion is supported by the observation that, when the *E. coli* expressing either WT or mutant Pol were grown at 30 °C, the vast majority of the WT and the mutant RTs were correctly processed and soluble.

The results we obtained when we monitored the behavior of the RT and Pol proteins in *E. coli*, taken together with the data showing that the purified mutant RTs unfold at a lower temperature than WT RT, provides strong support for the proposal that the mutant RTs are partially unfolded at 37 °C, which makes them more susceptible to degradation by PR. These data support our earlier conclusions that the degradation of the mutant RTs involves cleavage sites that are present in WT RT, but are protected from PR by the folding of the mature heterodimer. The data also reinforce the importance of understanding the impact of mutations in RT (and other viral proteins) on their susceptibility to PR; mutations that lead to the degradation of other viral proteins could also have a significant impact on viral fitness.

Material and methods

Preparation of the expression plasmids and growth of the E.coli strains

The *E. coli* expression systems used to generate the various forms of the HIV-1 RT have been described (Boyer et al., 1994,

2001; Hizi et al., 1988). The constructs that express the p66 subunit alone (leading to p66 homodimers), p51 subunit alone (leading to p51 homodimers), and the subunit selective p66/p51 (which creates heterodimers with the mutation in only one subunit) are based on the vector pUC12N and the proteins are constitutively expressed. The constructs are transformed into DH5α cells. The cells are allowed to grow overnight and are harvested the next day. Bacteria were collected by centrifugation for 5 min at 325 × *g*. Media was removed and the pellet was resuspended in PBS. For protein purification, 1 l of cells were used and purified as described below. For Western blot analysis, 4 ml cultures grown for two hours were used; the OD_{600} was measured and equal amounts of *E. coli* were pelleted gently by centrifugation for 5 min at 325 × *g*. The supernatant was removed and the pellet was vortexed and stored −70 °C.

The vector that encodes and expresses the p66 subunit and separately encodes and expresses protease was used to generate p66/p51 heterodimers with the mutations in both subunits. For the experiments described here, we generated a new plasmid that expresses the entire Pol coding region in *E. coli*. The Pol region was derived from the HIV-1 clone BH10 (Genebank #HIVBH102). A glycine codon located in p6* (nucleotide #1545) 22 codons from the beginning of the protease coding region was selected as the start position. PCR amplification was used to add a methionine initiation codon, and the sequence around the ATG was modified to create an NcoI site (CC ATG GGT AGA GAC…..). The same PCR reaction was used to add a HindIII site 3′ of the termination codon at the end of the IN coding region (nucleotide #4454). The PCR product was digested with NcoI/HindIII and cloned into the expression vector pT5, which is similar to the pET vectors. The vectors that encode both p66 and protease, and the Pol expression plasmids express HIV protease, which is toxic to the bacteria. These proteins are therefore expressed in the IPTG-inducible vector pT5m. The vectors were transformed into Rosetta 2(DE3) Competent Cells (EMD Chemicals). *E. coli* from an overnight culture were inoculated 1:80 into pre-warmed NZY/amp (100 μg/ml) for 2 h at 30 °C or 37 °C. The bacteria that expressed p66 plus protease, or Pol were induced with IPTG (Isopropyl-beta-D-thio-galactopyranoside at 2.5 mM final) for 2 h at either 30 °C or 37 °C. Bacteria were collected by centrifugation for 5 min at 325 × *g*. Media was removed and the pellet was resuspended in PBS. For protein purification, 1 l of cells were used and purified as described below. For Western blot analysis, 4 ml cultures were used; the OD_{600} was measured and equal amounts of *E. coli* were collected by centrifugation for 5 min at 325 × *g*. The supernatant was removed and the pellet was vortexed and stored −70 °C.

The various mutations were introduced in the RT sequence in these various constructs using the QuikChange Lightning Multi Site-Directed Mutagenesis Kit (Agilent Technologies: La Jolla, CA)

Differential scanning fluorometry

We used differential scanning fluorometry (DSF), (Niesen et al., 2007) to monitor the thermal stability and unfolding of purified RT. DSF was used to measure the uptake of a hydrophobic dye, SYPRO Orange (Invitrogen). The fluorescence of the dye increases when it binds to hydrophobic residues that are exposed as a protein starts to unfold. Because the fluorescence of the dye is low when SYPRO Orange is in an aqueous environment, the background fluorescence is also low. Purified mutant RT proteins and wild-type RT proteins were diluted to 0.25 μg/μl in 20 mM Hepes pH 8.4, 50 mM NaCl, 5 × SYPRO Orange in 100 μl. A temperature scan was conducted from 25 °C to 90 °C with a ramp rate of 1 °C/min. The SYPRO Orange fluorescence was monitored using a Stratagene MX4000 with excitation filters at 492 nm and emission filters at 610 nm.

Gel analysis of the recombinant RTs

Proteins were extracted from *E.coli* pellets that had been stored at −70 °C. The pellets were resuspended in 60 μl of 10 mM Tris-Cl, 1 mM EDTA, 25% sucrose, 5 mg/ml lysozyme, pH 8.0 and placed on ice 15 min. 82 μl of 50 mM Tris-Cl, 6.25 mM EDTA, 0.1% Triton X-100, 50 mM NaCl, 0.2 mM PMSF, 8U Benzoase (Novagen), 22 mM $MgCl_2$, pH 8.0 was added to the lysed bacterial pellet and placed on ice 15 min. Polymethyl sulfoxide (PMSF) was dissolved in ethanol at a concentration of 17.4 mg/ml (100 mM) and added to the buffer 15 min before the buffer was used. 180 μl of 50 mM Tris-Cl, 6.25 mM EDTA, 0,1% Triton X-100, 950 mM NaCl, pH 8.0, 0.2 mM PMSF was added and the lysates were centrifuged at 16,000 × *g* for 1 h at 4 °C. The supernatant was removed from the pellet and the OD_{280} of the supernatant was measured. Equal amounts of supernatants and pellets (based on the OD of the supernatants) of the bacteria that expressed WT and the RT mutant proteins were mixed with loading buffer (Final concentration of the loading buffer: 1 × NuPage LDS sample buffer, and 10 mM dithiothreitol, 50 mM ß-mercaptoethanol), aliquoted, and stored at −70 °C. The samples were heated to 95 °C for 5 min. Protein samples were fractionated on NuPage 4–12% Bis-Tris gels.

Purification of WT and mutant RT

The same purification procedure was used for the WT and the RT mutants. In each case, frozen cells (approximately 2 g) were thawed and homogenized with 4 ml of cold 50 mM $NaPO_4$, 50 mM NaCl, 0.75 mg/ml lysozyme, and 1.5 mM PMSF, pH 8.0. After a 25 min incubation on ice, 0.43 ml of 4 M NaCl was mixed with the extract. The suspension was sonicated 3 s × 30 s at 50% output with a 70% pulse. The maximum output of the sonicator was 350 W. Following sonication, the sample was centrifuged at 85,000*g* × 60 min. The supernatant was separated from the pellet and diluted with an equal volume of 66 mM $NaPO_4$, 300 mM NaCl, pH 6.8. The sample was then loaded onto a 1.5 ml nickel-nitrilotriacetic acid (Ni-NTA) metal-affinity column which was equilibrated with 50 mM $NaPO_4$, 300 mM NaCl, pH 7.0. After loading the sample, the column was washed with 50 ml of equilibration buffer. Next, the column was washed with 90 ml of 50 mM $NaPO_4$, 300 mM NaCl, 10% glycerol, 20 mM imidazole, pH 6.0. The RT was eluted with a 15 ml × 15 ml, 20 mM to 500 mM imidazole gradient with the other pH 6.0 buffer components present. Fractions of 1.5 ml were collected and analyzed by Coomassie stained SDS-PAGE. Fractions containing significant amounts of RT were pooled.

The pool was dialyzed against Q-B buffer (25 mM Tris acid/25 mM Tris base pH 8.3) over an 8–12 h period. The dialysate was centrifuged at 14,000*g* × 30 min. The supernatant was loaded to a 3 ml Q-sepharose column equilibrated with Q–B buffer. After loading was complete, the column was washed with 25 ml of Q–B buffer. A pH gradient consisting of 15 ml of the Q–B buffer and 15 ml of 50 mM HEPES/25 mM MES, pH 6.25 was run through the column. Samples were pooled after analysis with silver-stained SDS-PAGE. One-tenth volume of 2 M NaCl was added to samples which were then concentrated by centrifugal ultrafiltration using a unit with a 30 kDa cutoff. The concentration of the sample was measured by the Bradford method using bovine γ-globulin as the standard. All procedures in the purification were carried out at 4 °C.

Western blots

The proteins were fractionated on a NuPage 4 to 12% Bis-Tris gel (Invitrogen); and wet blot transferred onto Hybond-ECL nitrocellulose (Amersham). Blots were incubated in block solution (5% dry

milk in Tris-buffered saline-Tween) for more than 1 h. Primary antibody was diluted in 0.5% dry milk in Tris-buffered saline-Tween, and blots were incubated for 4 h to 24 h. Blots were washed 10 times for 6 min (1 h total). Secondary antibody was diluted in block solution, and the blots were incubated for 2 h to 4 h. The blots were washed five times for 6 min (30 min total), and the antibody was detected with either SuperSignal West Pico or Supersignal West Femto chemiluminescent substrates (Pierce). The individual mouse anti-RT antibodies have been described (Ferris et al., 1990); the anti-RT MAb mix was prepared using equal amounts of MAbs 19, 21, 42, 48, and 50. The mouse anti-RT MAbs were diluted 1:500; mouse anti-PR antibody (Exbio #10-302-C100) was diluted 1:2000. The rabbit anti-IN antibody (kindly provided by Duane Grandgenett) was used at a dilution of 1:10,000. With the exception of the data presented in Fig. 5, the Western blot data shown in the figures for the three anti bodies (to IN, PR, and RT) were done with a single batch of *E. coli* extracts. The experiments were repeated several times, with different extracts, and the data shown in the figures are representative. It was more difficult to get good blots/exposures in the Pol expression experiments, and in the experiments shown in Fig. 5, different experiments were done (with different *E. coli* extracts) with the antibodies to IN, PR, and RT.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2013.06.017.

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